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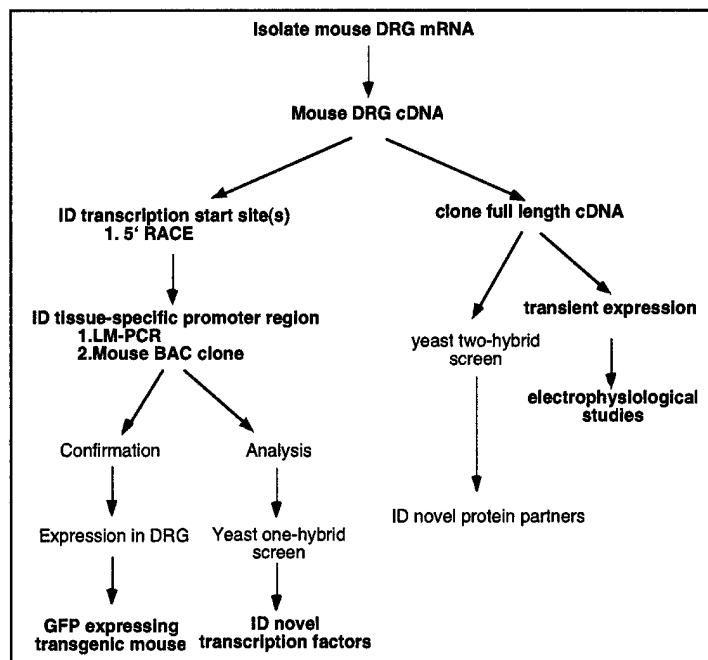
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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) The primary accomplishments of the previous funding period were: 1) RACE analysis of the 5' end of Nav1.8/Scn10a murine DRG cDNA confirmed the presence of one intron splice site in the ~200 bp 5'UTR of the Scn10a transcript; 2) Three BAC clones were isolated from a genomic library. All three contain <u>at least</u> the entire cds containing portion of the Scn10a gene plus the 5'UTR and an additional >4.0kb of upstream sequence; 3) Homologous recombination (conversion) of all three BAC clones to EGFP containing reporter constructs has been completed; 4) LM-PCR upstream from the putative transcription start site (5'RACE end) produced 4.0kb of sequence that may contain some/all of the elements required for the exquisitely controlled expression of Nav1.8, the Scn10a gene product; 5) The putative promoter appears to contain several known consensus binding sequences for transcription factors; 6) Putative neuronal-specific silencer elements may be present. Deletion analysis of the 4 kb promoter fragment indicates activating elements between ~ -3200 and -2500 bp. These latest findings will allow us to extend our analysis of the regulation of the Scn10a gene and efficiently focus our efforts during the final year of this project.				
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Introduction

The *Scn10a* gene product encodes a tetrodotoxin-resistant sodium channel (SNS/PN3) expressed exclusively in a subset of primary sensory neurons (e.g., dorsal root and nodose ganglia) believed to be involved in pain transmission (Akopian et al., 1996). Thus, it is important to understand mechanisms contributing to both the function of the protein and the exquisite specificity of gene expression. The overall research plan is detailed in the flowchart depicted to the right. Significant progress was made during the latest funding period on both the genomic (left branch) and proteomic (right branch) sections of the research plan.



Specifically, we have isolated three bacterial artificial chromosome (BAC) clones that contain the coding sequence for *Scn10a* and extensive 5'-flanking DNA that contains some/all of the *cis* regulatory elements that control expression of this gene. The BAC clones have been engineered to now contain the coding sequence for enhanced green fluorescent protein (EGFP) immediately downstream of the 5'-flanking sequence that will allow an analysis of *Scn10a* regulation in microinjected rat sympathetic neurons. In parallel, the putative transcriptional start site for *Scn10a* was identified by 5' rapid amplification of cDNA ends (RACE) which led to the isolation of 4.0 kb of genomic sequence immediately upstream of the transcriptional start site by ligation-mediated PCR (LM-PCR). Expression of EGFP protein was detected in rat sympathetic neurons microinjected with a fusion construct of this 4.0 kb genomic DNA to the EGFP gene. Deletion analysis of this 4.0 kb genomic sequence further localized essential *cis* regulatory elements. The BAC clones also contain this 4.0 kb of genomic sequence. Extensive sequence analysis of this 4.0 kb region and the flanking sequences contained on one of the BAC clones revealed consensus binding sites for essential transcription factors on the genomic sequence.

Body

1. Construction of Scn10a-Enhanced Green Fluorescent Protein (EGFP) Fusions in BAC (bacterial artificial chromosome) clones harboring the Scn10a gene

We reasoned that the Scn10 gene promoter was likely very large since other Na⁺ channel genes are regulated by promoters that span greater than 50 kb of genomic DNA. We, therefore, chose to screen a mouse genomic BAC library to increase the likelihood of isolating the entire promoter. Three BAC clones obtained from Incyte Genomics (#26462, #26463, and #26464) were identified in a PCR-based screen using oligonucleotide primers specific to exon 1 of the Scn10a gene. Restriction analysis of the clones shows that they are similar and carry extensive overlapping stretches of mouse genomic DNA (Figure 1.1). Extensive sequence analysis has been performed which confirms that the BAC clones contain extensive 5' flanking sequence to the Scn10a gene (Figure 1.2 and appended sequence).

Figure 1.1 Digestion of alkaline lysis miniprep isolated BAC clones with EcoRI

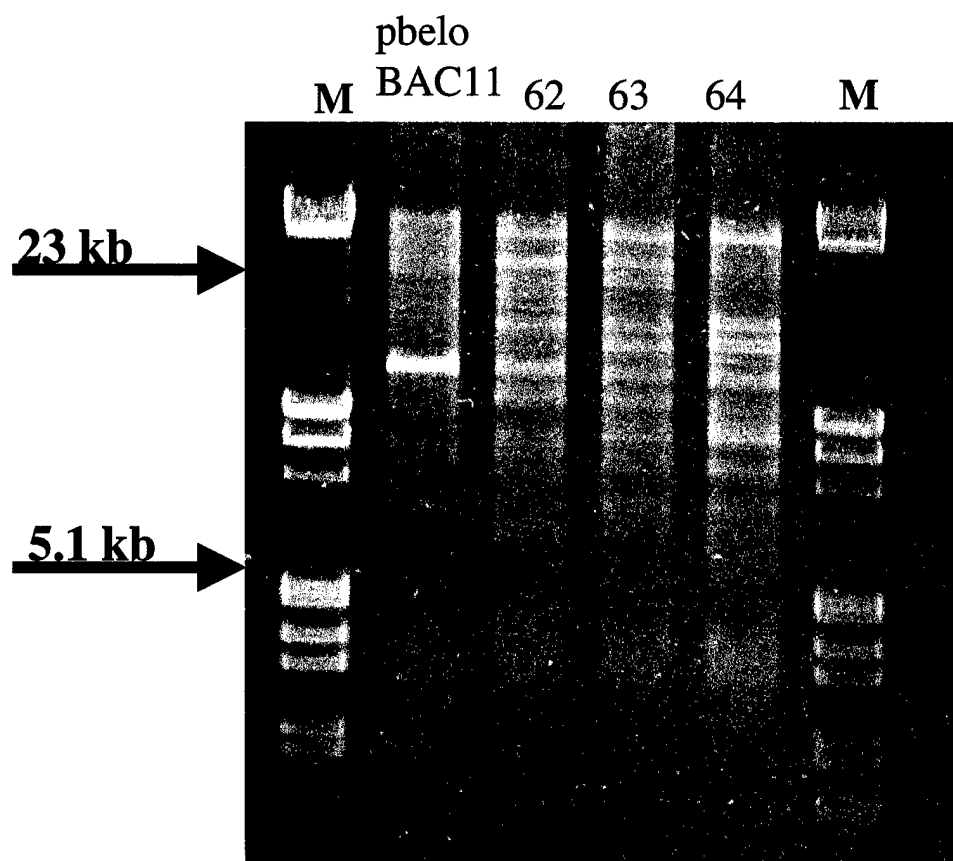
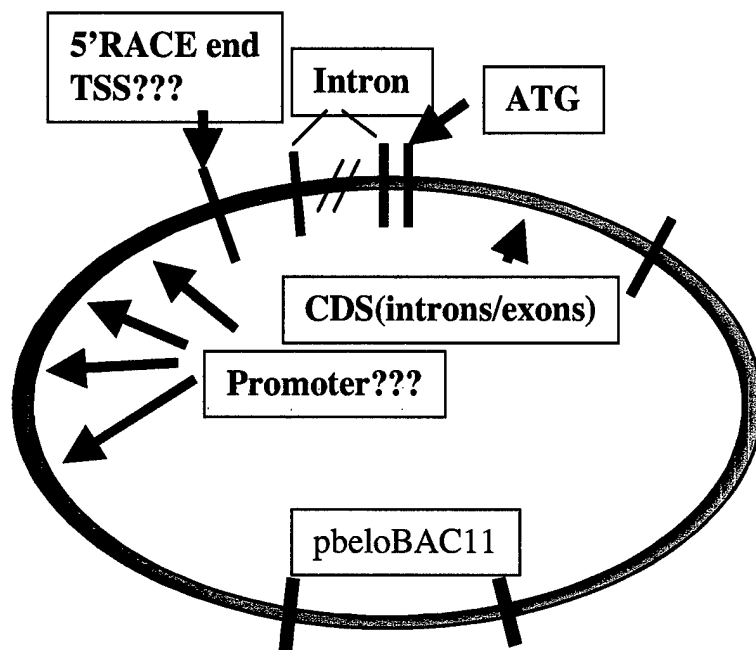


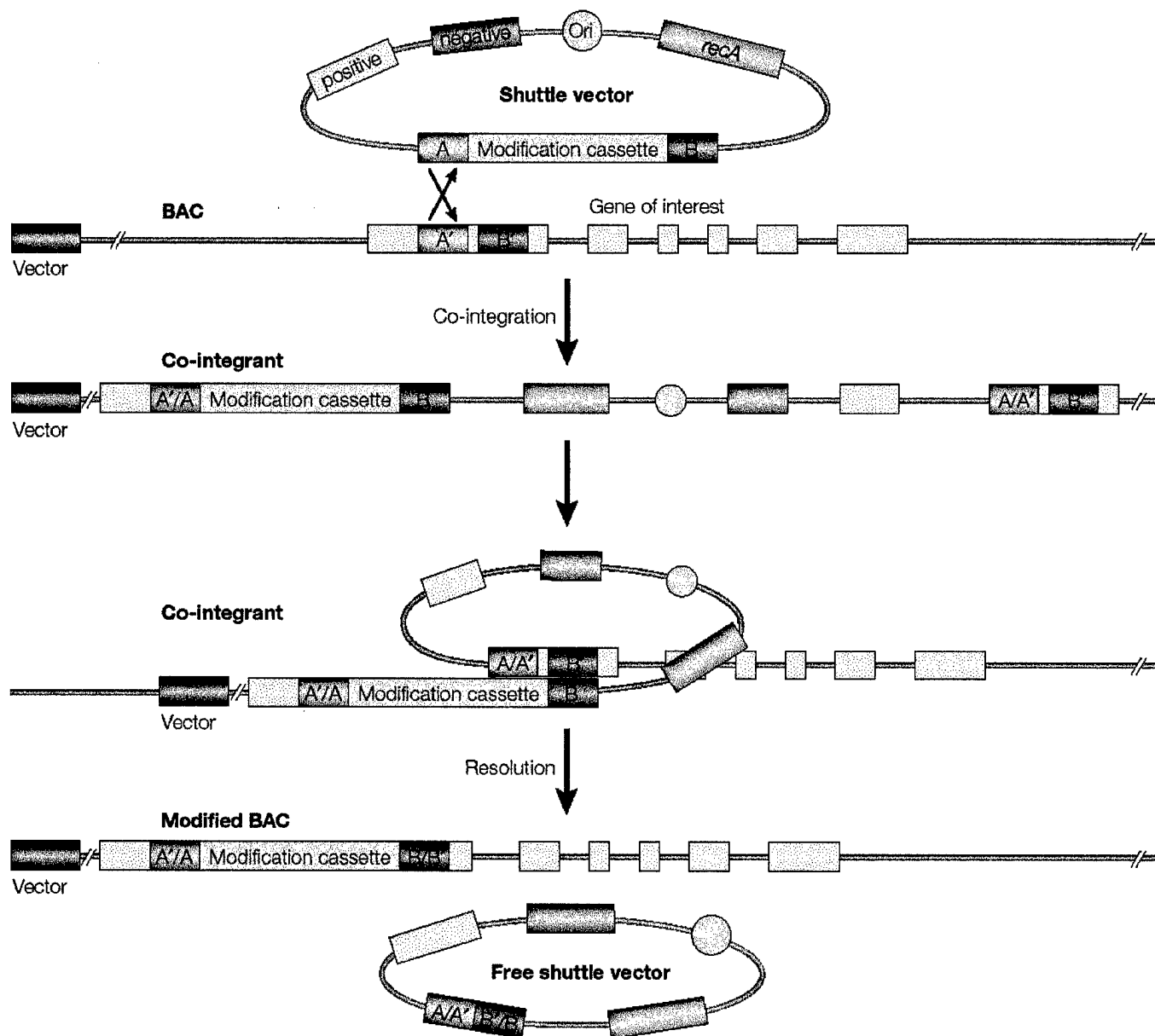
Figure 1.2 Direct BAC sequencing shows the presence of a large portion of the Scn10a gene in all three BAC clones.



Use 5' RACE data to design sequencing primers
Assess extent of genomic insert

We have modified the BAC clones by integrating the coding sequence for the enhanced green fluorescent protein (EGFP) downstream of the 5'-flanking sequence of the Scn10a gene contained in the BACs. This modification of the BAC clones will also allow us to examine the Scn10a gene promoter in transfected primary neurons. Expression of the EGFP gene under control of the Scn10a gene promoter is expected to provide a very sensitive read-out of this promoter's activity. The technique for inserting EGFP downstream of any DNA sequence contained on a BAC clone was first described by Yang et al. (1997) and the general scheme is shown in Figure 1.3 on the following page.

Homologous Recombination of BACs

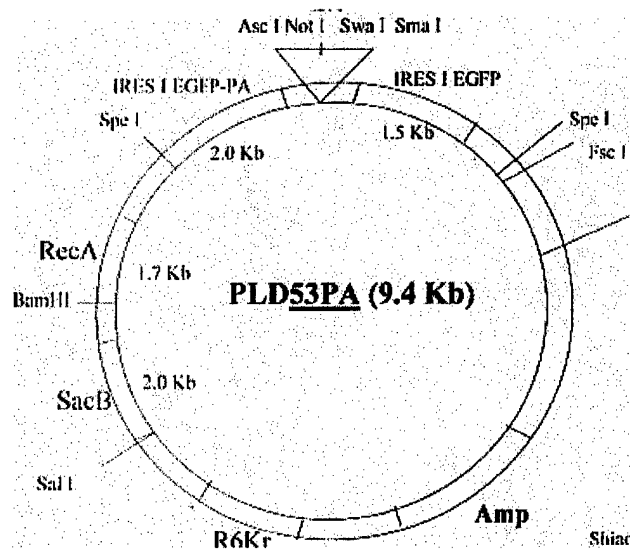


From Heintz *Nature Reviews* 2:861,2001

In this protocol, a specialized shuttle vector was first constructed *in vitro* that carries a 600 bp segment of the 5'-flanking sequence of the *Scn10a* gene fused to the EGFP gene in the shuttle vector PLD53PA (Figure 1.4).

This 600 bp subfragment was subcloned into the multiple cloning site of the shuttle vector via the NotI and SmaI sites incorporated into the forward and reverse primers. Homologous recombination between the 600 bp Scn10a fragment contained on the shuttle vector with the same

Figure 1.4 Creation of a reporter construct by recombination of a BAC clone



New version:
Homologous
recombination
shuttle vector

region contained on the BACs was performed *in vivo* resulting in co-integrates (Figure 1.6, next page). The co-integrates contain the 5'-flanking sequence of the Scn10a gene on the BAC clone fused to one of the two IRES-EGFP sequences of the shuttle plasmid. The co-integrates were resolved by utilizing the SacB gene on the shuttle plasmid to sucrose-counterscreen for BAC recombinants that have undergone a recombination event resulting in a final BAC clone containing a stably-integrated Scn10a promoter-EGFP fusion. Recombination can take place either between the two IRES-EGFP sequences or between the two 600 bp Scn10a regions contained on the co-integrates. Only recombination between the IRES-EGFP sequences will result in a final BAC recombinant containing the proper fusion (Figure 1.5).

Confirmation that the final resolved BAC clones contained properly integrated IRES-EGFP sequence was done by PCR using specific primers that were expected to yield a 3 kb PCR product with the resolved BAC recombinant as template if the fusion was present. If the fusion was not present, the expected PCR product would be 1 kb in size. The results confirmed that the BAC clones contained the correct fusion (Figure 1.6).

Figure 1.5 Construction of Scn10a-IRES-EGFP BAC Recombinants

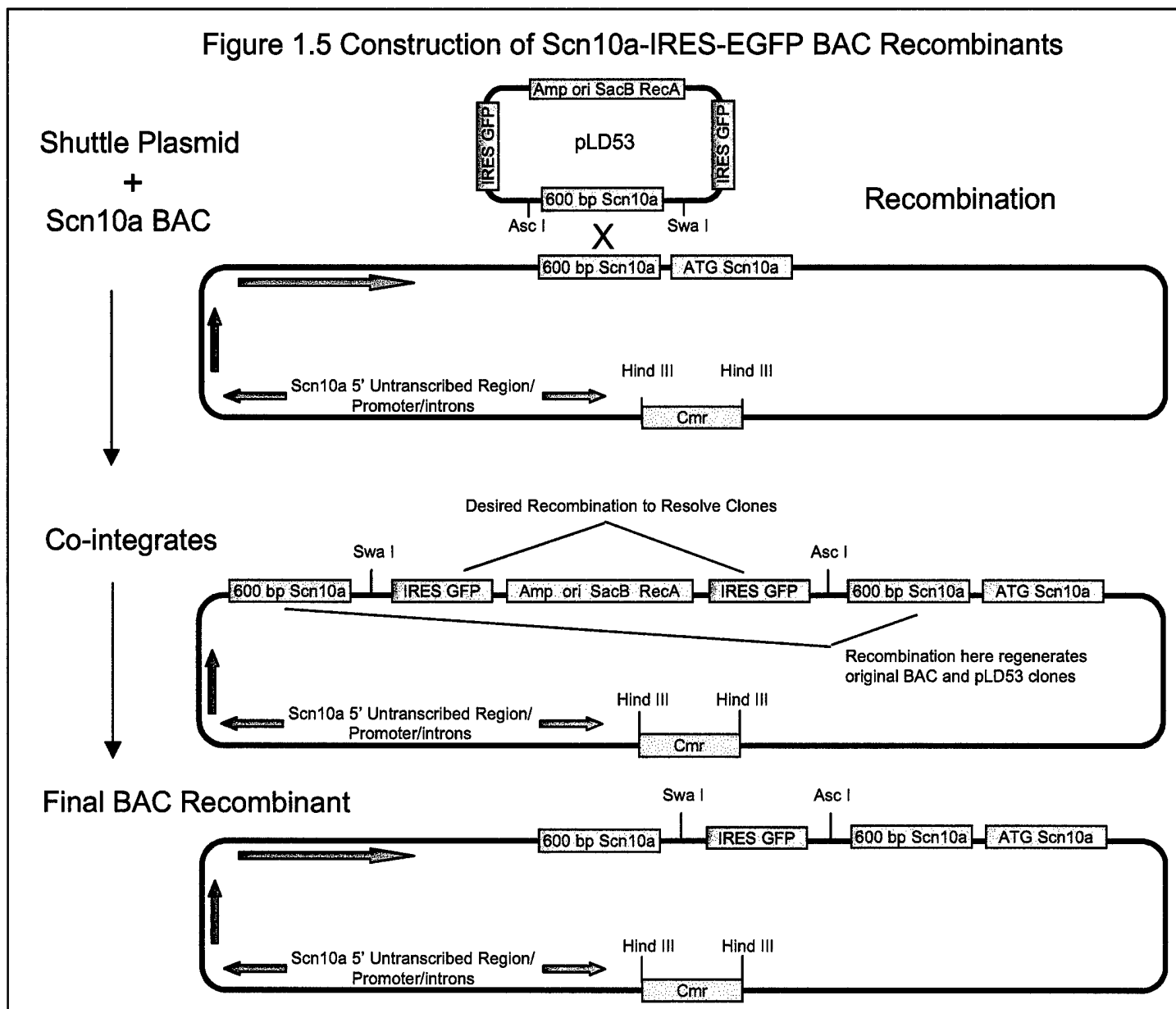
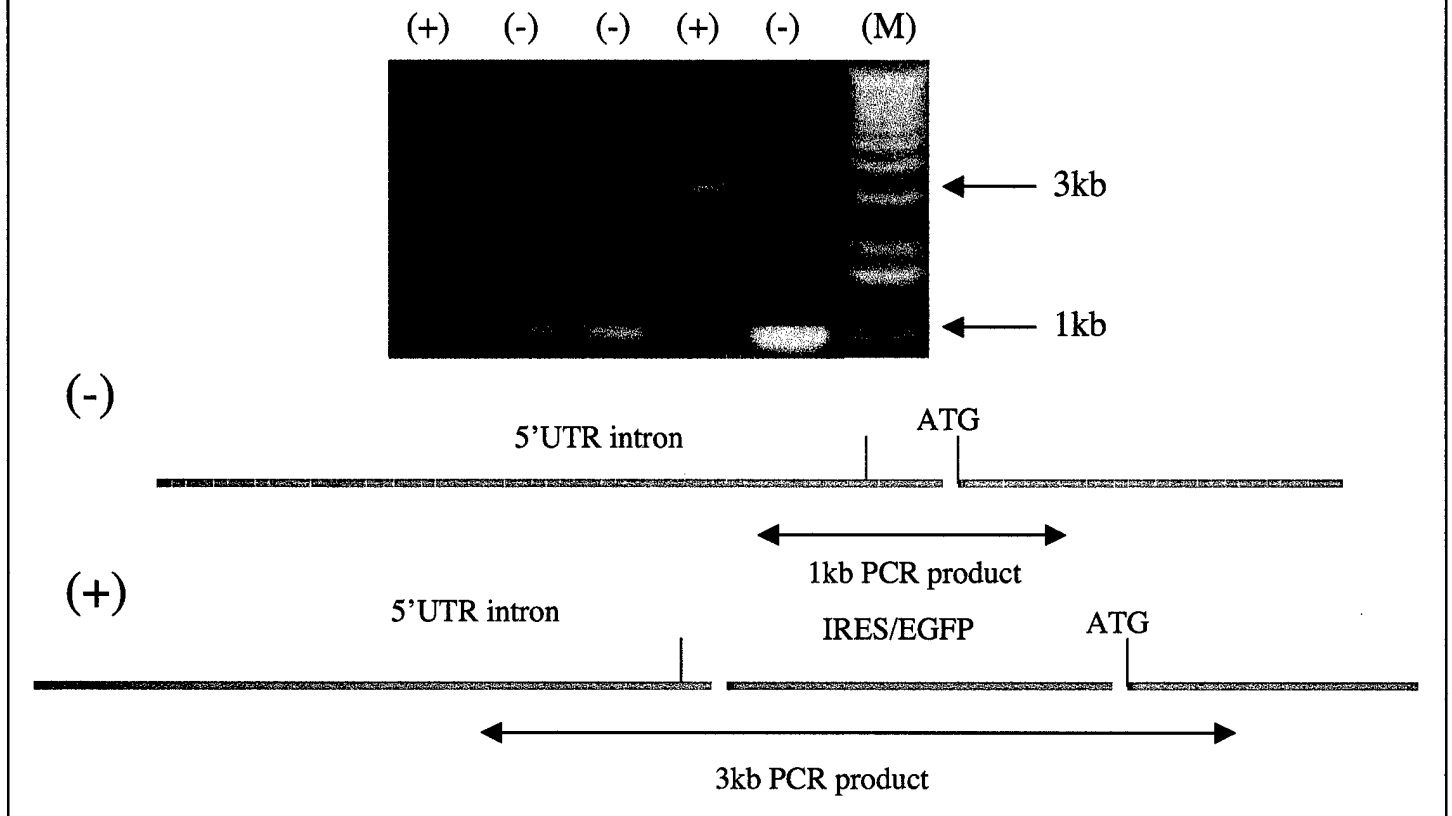


Figure 1.6 Homologous Recombination Results



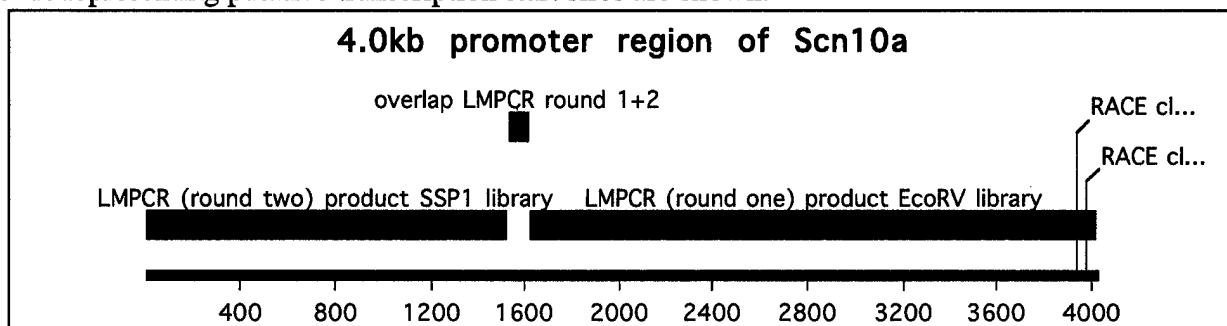
Reference

Yang, X.W., Model, P. and Heintz. Homologous recombination based modifications in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nature Biotechnology* **15**:859-865, 1997.

2. Expression studies with 4.0kb and 2.5kb LM-PCR products:

Two successive rounds of LM-PCR yielded sequence information corresponding to approximately 4 kb of genomic sequence flanking the 5' end of the *Scn10a* transcriptional start site. The sequence and structure of the two LM-PCR fragments are shown in figure 2.1 below. Sequences corresponding to each fragment and the region of overlap are indicated. The 2.5kb product of the first round of LM-PCR and the combined 4kb fragment were found in all three BAC clones by the PCR.

Figure 2.1. Schematic of 4.0kb fragment generated by two rounds of LM-PCR. 5' RACE ends representing putative transcription start sites are shown.



Sequence Range: 1 to 4032

>LMPCR_(round_two)_product_SSP1_library

10	20	30	40	50	60
ATTCCAGTTGCTGAGTGGAGAGAGCACTGTAGGGTCATGGAAGGACAGTGGGGAGGTCTG					
70	80	90	100	110	120
TTAGAGGTCTTGAAATTATATAGTGACCTCGCCATGATGGTGGTCTCAGAGATCGAGAG					
130	140	150	160	170	180
ATGATGTAATCAGGAGGACTCTAGGAATTCAAGTTAGAGGCCCCAGAAAGAGGGCTGTGG					
190	200	210	220	230	240
ACGAGGGACGGCTCTTGGATTACCTCTAGATGCTGGGCTTGTGAGTCCAGGCAAGCAGAG					
250	260	270	280	290	300
TGTTCCTTGGAGAGGCTTCTCTGGGGGAGGATCATTCTGAGCAGGGCACAGGCACAGAAAT					
310	320	330	340	350	360
CATTAGTCCATCTGTAAACATGTCTGAGATGTTAGTGGAGTGTCCATGAAGGGAATTCA					
370	380	390	400	410	420
GGCTTCTACCACATTAGTGTATATTTAAATCTGACACCAGGAGAGAGATTTATGATGGAG					
430	440	450	460	470	480
CTGACAGACTCCGGTGCCATGTCAGGTAGGTGACTGAAGCCCTGGGGAAGGAGAGGCGTA					
490	500	510	520	530	540
GGATGGAATCTTAAAACGATTCTCCAATACTTCCAGGTGGCAGAGGAGGAGGCAGCCCA					
550	560	570	580	590	600
GGCCAGAGAAGCTCCTCTGAAAACAGAAGTCAAGAGGGTGGAGTGTGGTGCAAGGACCAT					
610	620	630	640	650	660

GCAGCTAATCCTGCGGAGCCCCCTAGGATGAGAGCGCCAGAGAGGAGACACATGACACAGG

670 680 690 700 710 720
GAGACCAGTAGAAACCTGTTAAGATTCCGGGTGTCTCAGGACTGCCTCTGGATGCACACT

730 740 750 760 770 780
TCTTCCTTCTTGGGAAGTTACTTTTCTGTCACTGTGATGAAATACCTTAACCAAGGTGAC

790 800 810 820 830 840
TCAAAGAAGAGAGGGTTTATCTGGGCTCACGGGTCCAGAGGTAGAGGAACACATGGAGAT

850 860 870 880 890 900
CGTGGTGGGGAACCAAGTGTAGCAGGCAAGCATGGTGGCTGGGGCTGAGGCTGAGAGCTTA

910 920 930 940 950 960
TATCTTTGTCTGTATACAGAAAGCAGAGAGAGCCAACTGGGAATGACTTGTGGCTTTTGG

970 980 990 1000 1010 1020
AACCTGAAACCTGTCTTCGGTGACATGCTCCCTCCAGCGAAGGCAATGCCTCCTCAAAC

1030 1040 1050 1060 1070 1080
CCCCAAAGGGCACCACAACTAGGAACCAAGCACTCAGATGCCCCGAGACTATGAGCGACA

1090 1100 1110 1120 1130 1140
TCTCCTTCAGATCACCACACTTGGGTACACCCATTCTCCTGTCTCATCCAGTTTGCTCTT

1150 1160 1170 1180 1190 1200
CTGGAAGGGTGGTGAGAGGGATGACAGCTAGTGACAAGTTGGAGAGACTTTAGAATAATT

1210 1220 1230 1240 1250 1260
GCCATCACACAAAGCCTACCCTATCAGTTAGTGGCTGGCACGCTATCCCAACAGCTTGAG

1270 1280 1290 1300 1310 1320
TCTGAACTTGCCAGAAATGGCCTCCGTCTCACCTCTCCCAGGCTCCCCAGCACCCACAGG

1330 1340 1350 1360 1370 1380
TGCCCTCCCCCAAGACCTGACATCATCGGAGCACTGAAGAGATGCCTCCTCTGCCCCCTT

1390 1400 1410 1420 1430 1440
CTCCCCTGGTCTGATTGCTACCAGGCAGCTGATCCACATGCCCTGCTCCAAGTTTGACCC

1450 1460 1470 1480 1490 1500
CAGTCAGCAGGCTTCTCTGAAGAAGAGGGTCTGTTAGCATGACACACAGCATTTCCCATG

>overlap_LMPCR_round_1+2

1510 1520 1530 1540 1550 1560
CAACAGAACCTTGAACCTAGGACAGAACTCAAGATATCAACGTGACACACACATGTACA

1570 1580 1590 1600 1610 1620
TGTACCCCTTACACACCTGAACGTGCATATACACACGTACACTTGTACACACACTAAAATA

>LMPCR_(round_one)_product_EcoRV_library

1630 1640 1650 1660 1670 1680
ACTGTGGTGCCAGGAGTGATCAGGGTACTGAGGTGACAGTCATTCAACAAGGCTTGCCCTTT

1690 1700 1710 1720 1730 1740
CTCCACGTGACACACTTTGACCTTACCACCTGTTTCCACCTTGCTCTGGCATTTTAAAAA

1750 1760 1770 1780 1790 1800
CATGACATTTTGTAAATCTTTGAATTTTTTTGAGACAGGGTCTTACACTACAGCTCA

1810 1820 1830 1840 1850 1860
AGCTGATCTTGGGTTTGACGAATCCTCTTCCCTCGACCACCCCCCAACTAGGATGTGA

1870 1880 1890 1900 1910 1920
 GCTGCCATGCCCAGTTTGTACTCTTTCCAGATGTTTGTATTTTATTCTGTATGTATGAGTG
 1930 1940 1950 1960 1970 1980
 TTCTCACTGTATGTATGTCTGTATATGCACCATGTAGAGCCCCAAAAGCAGTTGCTGAATG
 1990 2000 2010 2020 2030 2040
 CTCCGGAGCTTGGAGCTGTGGGTGGCTGTGAGCTGCCACGTGGGGTGCCAAGAATAGAAC
 2050 2060 2070 2080 2090 2100
 ACAGGTCCTCTGCAAGAGCATCGAATGCTCTTAACCACTGAGCTATCCCTCCAGGTATTA
 2110 2120 2130 2140 2150 2160
 AAACATAAAATAAGCTTGACTTTTTACTCTGACATAATTTAAATTCACAAAAAGTTTTTA
 2170 2180 2190 2200 2210 2220
 AAAAAACGTAACAGCTTCCGTATACTCCCAATCCCATTTCCCCAGTTAGGATATTCTTTA
 2230 2240 2250 2260 2270 2280
 ACCATAGTACATTGTCAAATGAGAACTAACATTCATACAACACGACTGATTTTGGTGA
 2290 2300 2310 2320 2330 2340
 AAATCCTATTTGGAGTACACTACCTTTGACTGTGATTTCTTTTGCAGTATGGCCAGGC
 2350 2360 2370 2380 2390 2400
 TAGCCTGCAACTATTTATCATTATTTATCCCAGGGTGACTTGAACCTAGGGCAATTCTTC
 2410 2420 2430 2440 2450 2460
 TACCTCAGCTCTGCCCCACTCCCACCTCAACTCCAGTCCTGGGGTTACAGGAGCAAGCC
 2470 2480 2490 2500 2510 2520
 ATCAAGTTCTATAACATTTAATACACAAGGACACTGGTTAACTCAGAAGGACCTAAATT
 2530 2540 2550 2560 2570 2580
 AGCATAAGACTATGGGGACCAGAGAAGTGAGAAGTGAGGACAGGGGAGGAGGGCAGGGGA
 2590 2600 2610 2620 2630 2640
 GGGGAAGATGGGAGGAATGATGGGAAGAGAATGAGAGAAGGCAGGGAGGGAGGAGAAGG
 2650 2660 2670 2680 2690 2700
 CCAGTGAAGGGAGAATGGGAAGGGAGGGAGTTGAGAGAAGGCAGGATCGGGAGCCATAGA
 2710 2720 2730 2740 2750 2760
 ATGTCTGTAGGAAACCATCAAAGGCATTTAATTTAATAAAGCAACCAGGATTGTACATAA
 2770 2780 2790 2800 2810 2820
 TTCTACTGTGTACATAAAACACTCAAGTTTTGGGAGCAAGAATTTTAGCTTTCCTTCCC
 2830 2840 2850 2860 2870 2880
 CTGCCCCCTTTATGATTCACTCTCTGCTAGAAAAAGTGGAGCCTTGCAGGGTGTGGTGGTG
 2890 2900 2910 2920 2930 2940
 CACGCCTTTAATTCCAGCGTTTGGGAGGCAGAGGCAGGTGGATTCTGTGAGTTCCCGGT
 2950 2960 2970 2980 2990 3000
 CAACCAAATCTCCATAGTATGATCCTTCGTGGAATACCGGCCAACCAACAAGCAAACAA
 3010 3020 3030 3040 3050 3060
 ACAAAACAAAAATCCCAAACAAACCCACCCCCACCCAAATAGAGGGGATTATTGACTCAA
 3070 3080 3090 3100 3110 3120
 AGAAGCCAATAATTTTGTAGTTGGTTTGGGACATTTGAGTAAATGAAGCTGTAATGGGCAA
 3130 3140 3150 3160 3170 3180

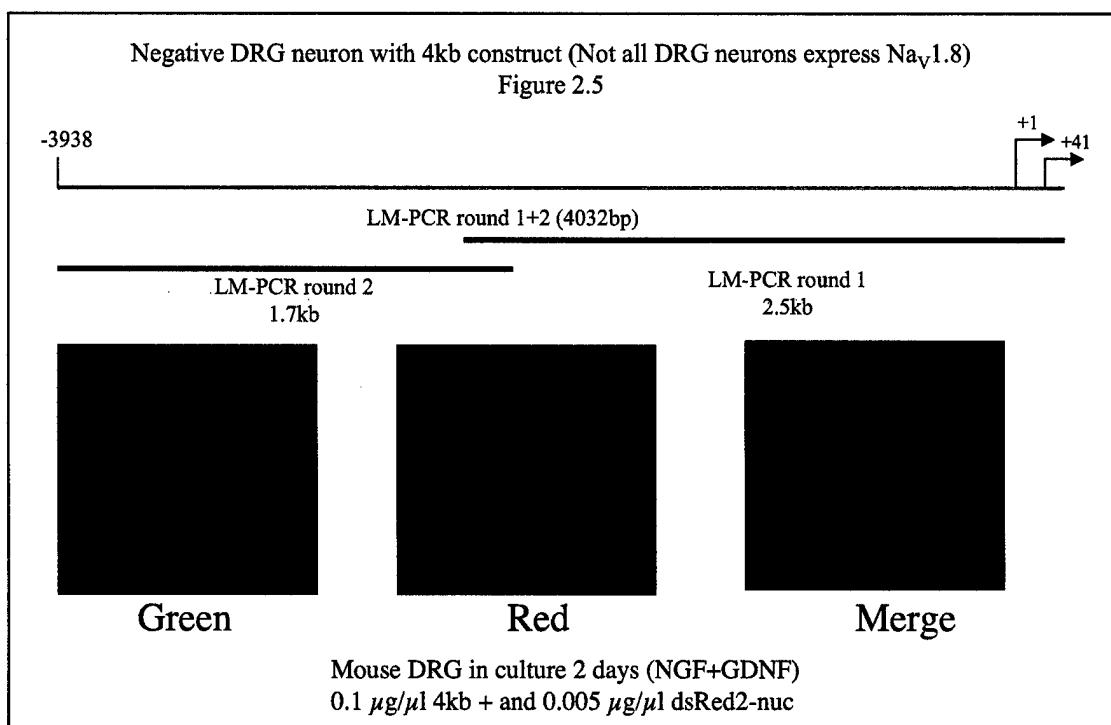
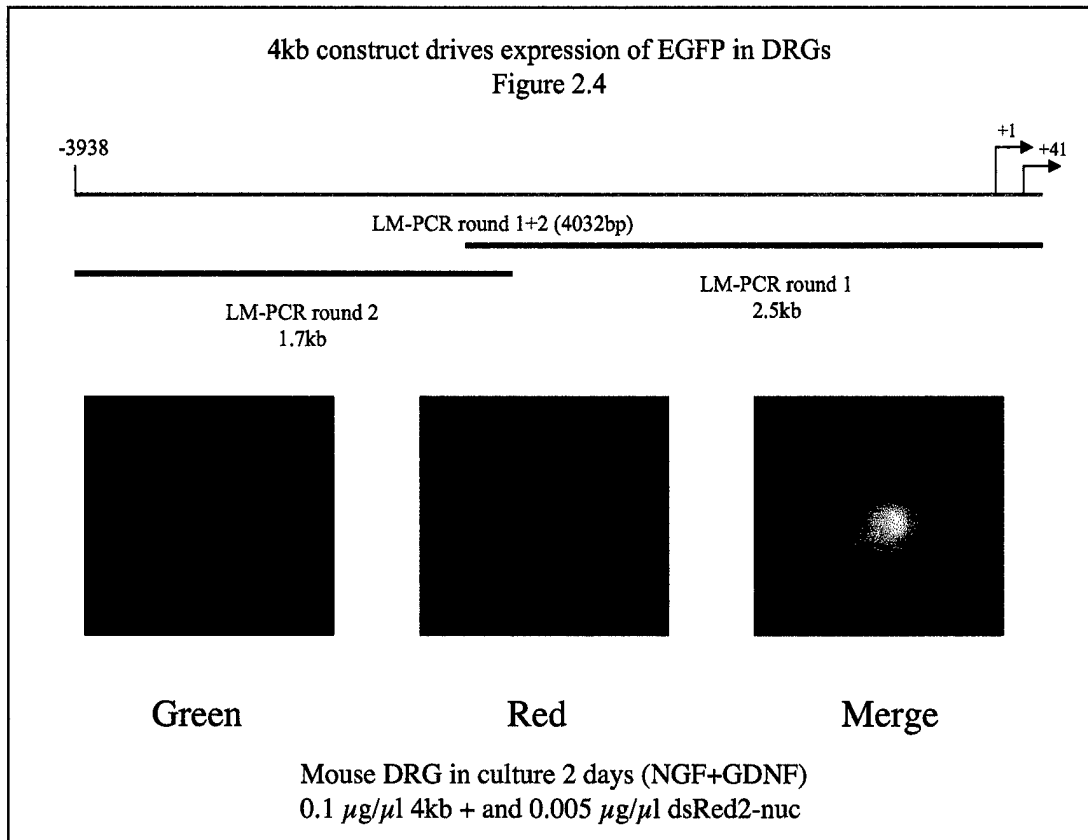
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GCATGGGCCCTCGACAGTTTCCTGCAGTATAGCATGGCTTCCTAAGGCTGCGTGGGTTGC
      3190      3200      3210      3220      3230      3240
ACTGTTACGGAGGGCTCAGCTCAGACAGGGGGTTCCTGTGCAACCTCCTTTCTTATGGT
      3250      3260      3270      3280      3290      3300
CCCACAACCCACAGATAGGGCACTTTCCTACCCAGCTCCCTTCTCGGCTCTCACTGGG
      3310      3320      3330      3340      3350      3360
GTCGGAGAACATTTTGTTCAGCATTTTCATCTGAAGCCACGGTTTCACATCATCAAGTC
      3370      3380      3390      3400      3410      3420
TGCAAAAAACCGTTCACAAACCACACCAGAACTTCTCGGTAAAGAACTCCTAAGACCAA
      3430      3440      3450      3460      3470      3480
GAGGAGACTGGGTAGATTGTTTTTAATTGTTTCTTTTGTCAAAGGGGGACAAAACAC
      3490      3500      3510      3520      3530      3540
GCTTTGGTGAGTGCGAGTGTATTCTGGGACACAAACCCAGAGTCTGGAAGGGAGCATT
      3550      3560      3570      3580      3590      3600
CAACGGGTGCTGCTCTGCCACGCAGGGGCAGCGGTGGGACTCAGCCCATCCTGCTAAGGA
      3610      3620      3630      3640      3650      3660
CGGGCAGCCTGAGCCAGGCTTGGGAGTCTGTCATGGCTGCCAGACGAATCATTATCTAAT
      3670      3680      3690      3700      3710      3720
TGCAGCCTTTTCTCTTCCTTAGGTTTCAGCAGGTCCCGAGAGAGCATTTAAATCGCATT
      3730      3740      3750      3760      3770      3780
TACTACTTTACCATCTAATCACACATAAGCCTCTCCCTATACCCTCCACCCTCCTCCAT
      3790      3800      3810      3820      3830      3840
TCAGAGTGTAATTCTGGAGCCCATCCAGCAAGCAGGGTGGAATCATGACGGGAAATGG
      3850      3860      3870      3880      3890      3900
GAACGGCGCCACGAAGGCGTGATTTCCTTGTAGATCCTTGAGTGATGGACGGGTGAGGTT
      3910      3920      3930      3940      3950      3960
TCCGTCAGGCAAGCCAGCCACCTTCGTGGAGGAGCCCCGGACAAGTGTAAGTTTCGCAG
      3970      3980      3990      4000      4010      4020
AGCTGGGGTCTCCAGCTTACTTCTGCTAATGCTACCCAGGCCTTTAGACGGAGAACAGA
      4030
TGGCAGATGGAG

```

The PCR products corresponding to these regions were clone into the pEGFP-1 vector from Clontech (see figure 2.2 below). This vector contains the coding region of the enhanced green fluorescent protein down stream from a multiple cloning site. The vector allows the analysis of sequences for promoter activity by their ability to drive expression of the EGFP protein product.

Figure 2.4 shows cells expressing EGFP following injection. Figure 2.5 shows an example of a successful injection, as viewed by dsRED production, with no EGFP production. The expression of *Scn10a* in only a subset of small diameter neurons in DRGs may account for the failure of this construct to express in all injected cells.



Injection of all constructs into sympathetic neurons isolated from superior cervical ganglia failed to produce visually detectable levels of EGFP. The positive control nuclear directed dsRED construct produced red nuclei in all cases. Scn10a is not expressed in these neurons and therefore this experiment serves as a negative control. Additionally, reporter constructs were electroporated into the human neuroblastoma cell line SK-N-SH. No green cells were visible even after three days in culture. Separate electroporations with SK-N-SH cells and a CMV driven GFP construct produced green cells.

Deletion analyses of the 2.5kb and 4.0kb fragment have begun. Six deletion constructs (three from each end) have been cloned and await analysis. The primer scheme is shown in figure 2.6. Constructs were generated by PCR with deletion and anchored primers from each end. Fragments were cloned via restriction endonuclease sites incorporated into the primers. Three deletion fragments of the 5' end of the 4.0kb fragment have also been generated by the PCR and cloned into pEGFP-1. These constructs designated S, M, and L were generated by designing primers to various positions of the parent 4.0kb fragment as shown in figure 2.6. The PCR was performed with an anchored primer at the 3' end of the 4.0kb sequence (Not Shown). Preliminary injection experiments performed as described above with the M or medium sized construct produced visibly green cells. The injection of the L (large) and S (small) fragments have not been performed.

Figure 2.6: A. 5' and 3' deletion strategy and results for 2.5kb LM-PCR round one fragment:

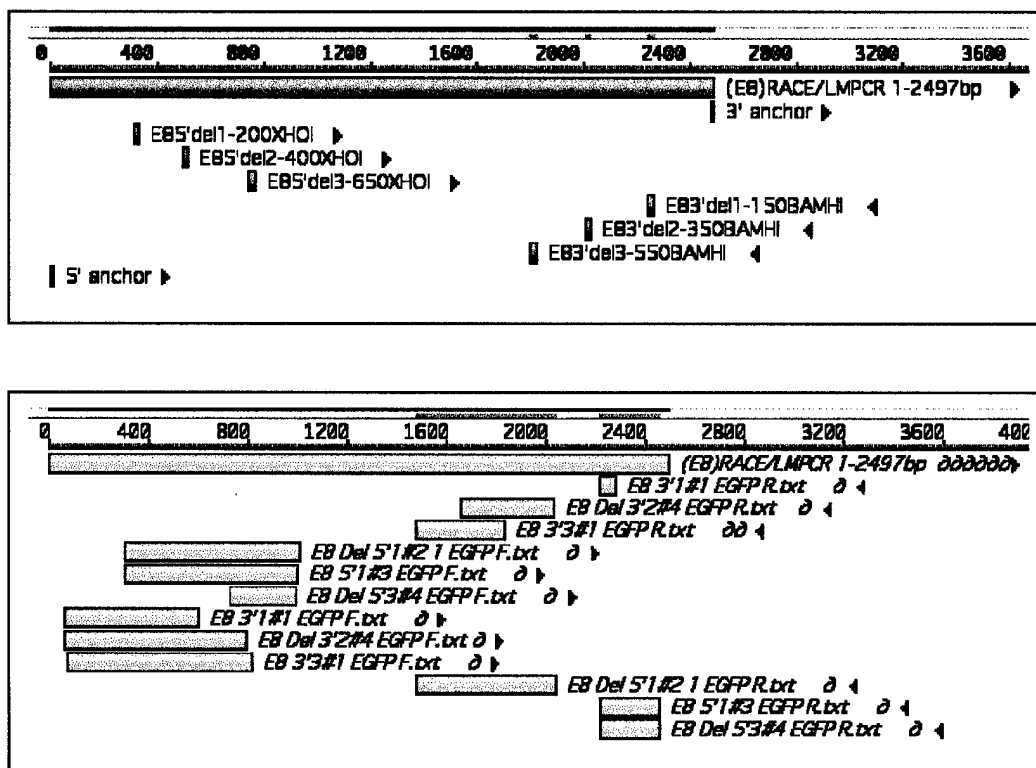
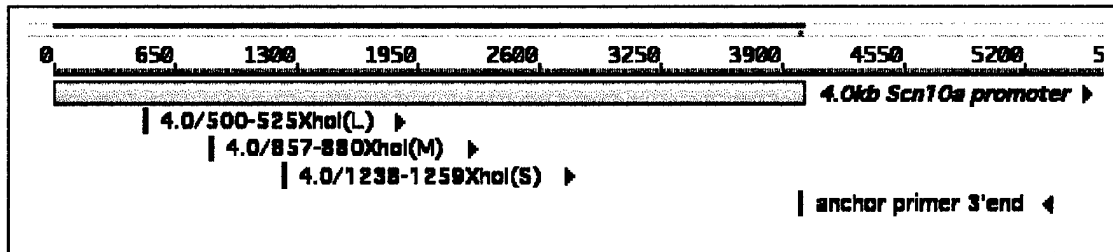
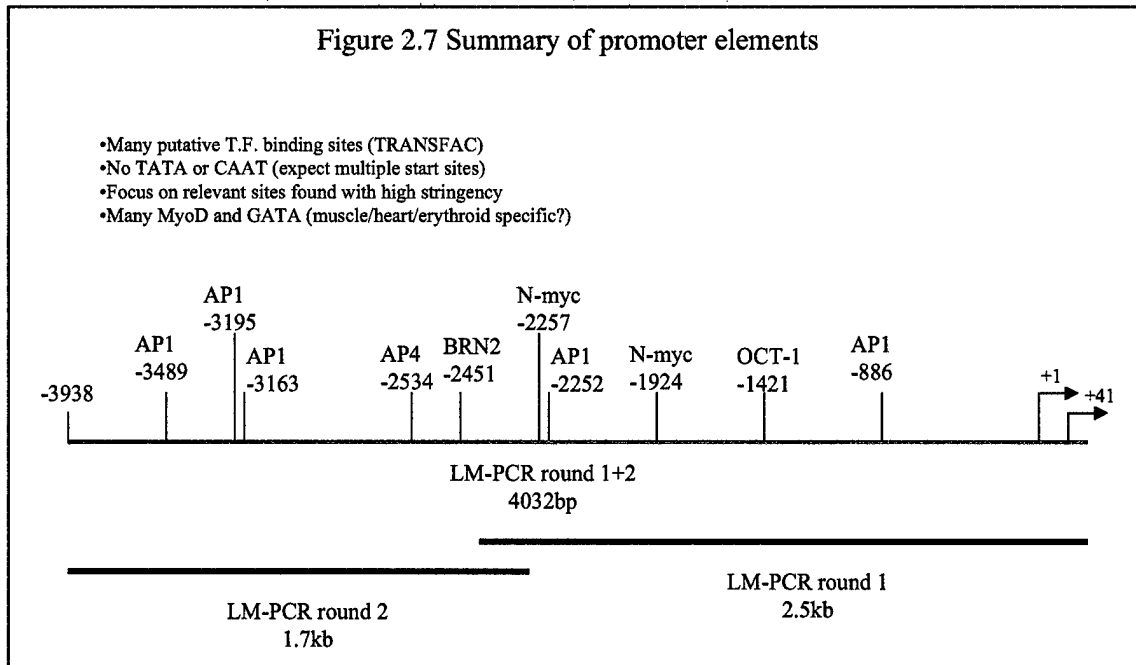


Figure 2.6B. 5' Deletion strategy for 4.0kb deletion constructs.



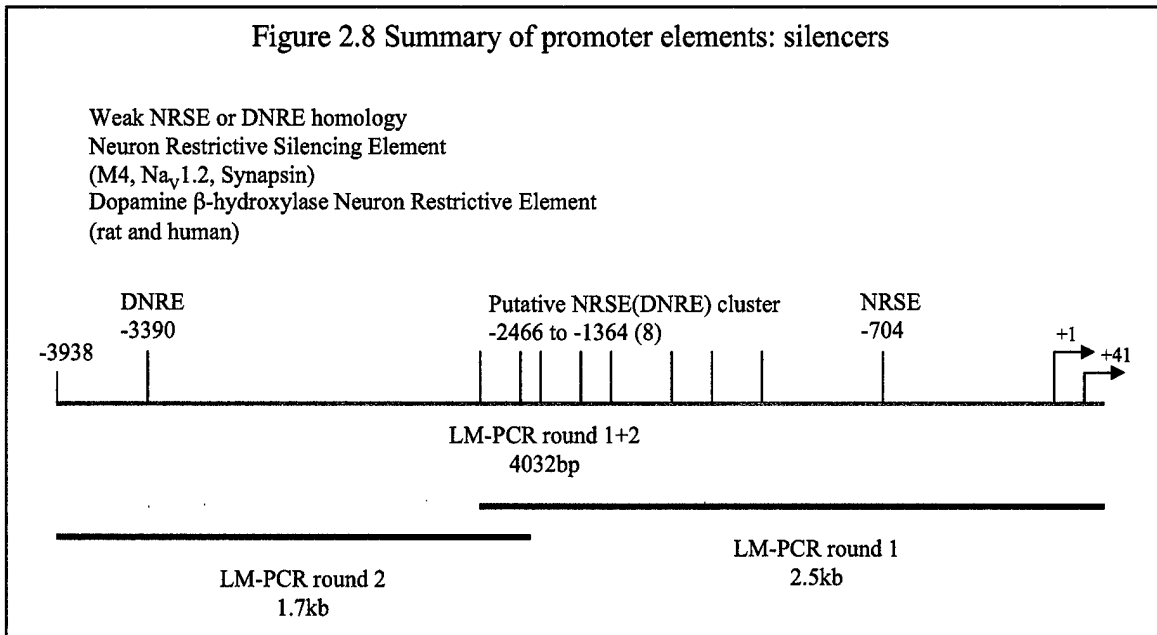
The results of this portion of experiments indicate that important factors involved in the expression of the *Scn10a* gene may lie in the region from the M construct to the point of origin for the 2.5kb fragment containing construct generated from the first round of LM-PCR. Sequence analysis of the entire 4.0kb fragment is shown in figure 2.7:



Our sequence was compared to the TRANSFAC database. This database searches input sequences for putative transcription factor binding sites. The results were extensive and therefore only a partial list of potential sites is shown in figure 2.7. This list includes several factors involved in expression of other neuron specific genes such as AP1, AP4, BRN2 and Oct1 (both POU factors) and N-myc.

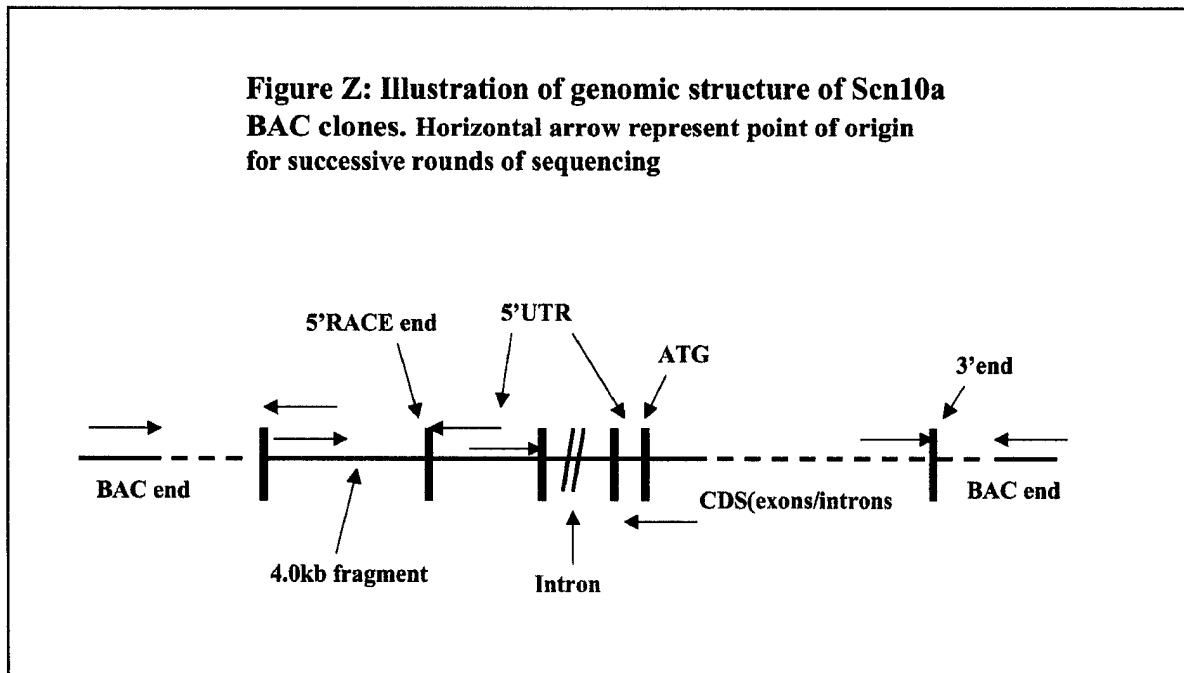
Figure 2.8 shows the presence of several sites with weak homology to a variety of silencer elements such as NRSE (Neuron restrictive silencer element) and DNRE (Dopamine beta-hydroxylase neuron restrictive element). These elements have been shown to be potent silencers capable of completely abolishing expression of genes carrying these sequences. The expression of genes associated with such sequences can occur only if the silencer protein is not expressed in a particular cell type.

Figure 2.8 Summary of promoter elements: silencers



Bacterial artificial chromosome direct sequencing has continued with a focus on clone #26463. This clone has been partially sequenced following the strategy outlined in Figure Z below. The illustration includes the known structure of the genomic fragment and shows, via horizontal arrows, the direction of successive sequencing reactions. The goal has been to span unknown region within the BAC constructs. The procedure has been successful in verifying the presence and sequence of the entire 4.0kb LM-PCR derived fragment. Sequence data from reactions with primers directed out from the 5' and 3' most coding exons have confirmed the presence of all coding exons for the *Scn10a* gene on the BAC clone #26463. The regions between the BAC ends and the start of the known *Scn10a* sequence have not been fully determined as these regions appear to be larger than the amount of sequence obtained thus far.

Figure Z: Illustration of genomic structure of *Scn10a* BAC clones. Horizontal arrow represent point of origin for successive rounds of sequencing



Additional information on the structure of the BAC clones is presented as Appendix material.

Key Research Accomplishments

1. RACE analysis of the 5' end of Na_v1.8/Scn10a murine DRG cDNA confirmed the presence of one intron splice site in the ~200 bp 5'UTR of the Scn10a transcript.
2. Three BAC clones were isolated from a genomic library. All three contain at least the entire cds containing portion of the Scn10a gene plus the 5'UTR and an additional >4.0kb of upstream sequence.
3. Homologous recombination (conversion) of all three BAC clones to EGFP containing reporter constructs has been completed.
4. LM-PCR upstream from the putative transcription start site (5'RACE end) produced 4.0kb of sequence that may contain some/all of the elements required for the exquisitely controlled expression of Na_v1.8, the Scn10a gene product.
5. The putative promoter appears to contain several known consensus binding sequences for transcription factors. In addition, putative neuronal-specific silencer elements may be present.
6. Deletion analysis of the 4 kb promoter fragment indicates activating elements between \approx -3200 and -2500 bp.

Reportable Outcomes

1. Ikeda, S.R., King, M.M., Aronstam, R.S. and Puhl, H.L. Cloning and expression of cDNA encoding a tetrodotoxin-resistant (TTX-R) sodium channel (Scn10a) from mouse dorsal root ganglion neurons. *Experimental Biology Meeting*, 2001.
2. Puhl, H. L., King, M.M. and Ikeda, S.R. Identification of the promoter region of the mouse scn10a gene encoding the tetrodotoxin-insensitive voltage gated sodium Channel Nav1.8, *Soc. Neurosci Abstr.* 2002, in press.
3. Puhl, H. L., King, M.M., Aronstam, R.S. and Ikeda, S.R. Cloning and functional characterization of mouse cDNA encoding a tetrodotoxin-resistant (TTX-R) sodium channel (Scn10a). *Soc. Neurosci. Abstr.* 2001.

Conclusions

Four major objectives were achieved during the previous funding period that extend our understanding of the regulation of the Scn10a gene.

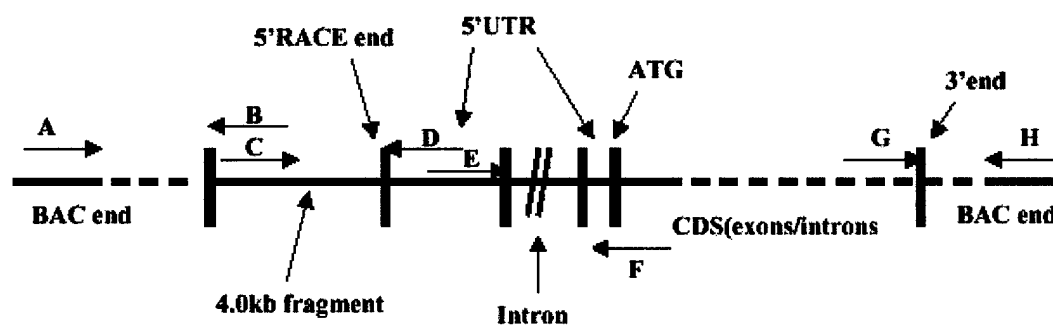
1. The three BAC clones containing the entire coding sequence of the Scn10a gene were converted into EGFP reporter constructs by homologous recombination of the EGFP coding sequence downstream of the 5'-flanking sequences contained on each BAC. These constructs will be analyzed by intranuclear injection of rat sympathetic neurons to determine whether essential *cis* regulatory elements for Scn10a are present.
2. The putative transcriptional start site for the Scn10a gene was determined by RACE analysis beginning at the ATG start codon. The presence of an intron splice site ~200 bp upstream from the ATG codon was found which suggested that the promoter region for Scn10a was interrupted by at least one intron of undetermined size. The genomic sequence immediately upstream of the RACE product was identified by LM-PCR which produced 4.0 kb of sequence that may contain some/all of the *cis* elements required for expression of Scn10a.
3. The 4.0 kb putative Scn10a promoter was fused to the EGFP reporter gene and injected into the nuclei of rat sympathetic neurons. The detection of EGFP expression in these neurons indicates that some/all of the *cis* elements comprising the Scn10a promoter are present within the 4.0 kb sequence. An EGFP reporter containing a deletion of the 5'-end of the 4.0 kb sequence (-3200 to -2500) did not express EGFP in rat neurons. This shows that essential activating *cis* elements are present within the -3200 to -2500 region. Sequence analysis also indicates the presence of consensus binding sites for transcription factors. The BAC-EGFP reporter constructs described above contain the 4.0 kb genomic sequence.
4. Extensive sequence analysis of one the BAC clones was done. This will complement the efforts described above to localize essential *cis* elements for the Scn10a gene.

These latest findings will allow us to extend our analysis of the regulation of the Scn10a gene and efficiently focus our efforts during the final year of this project.

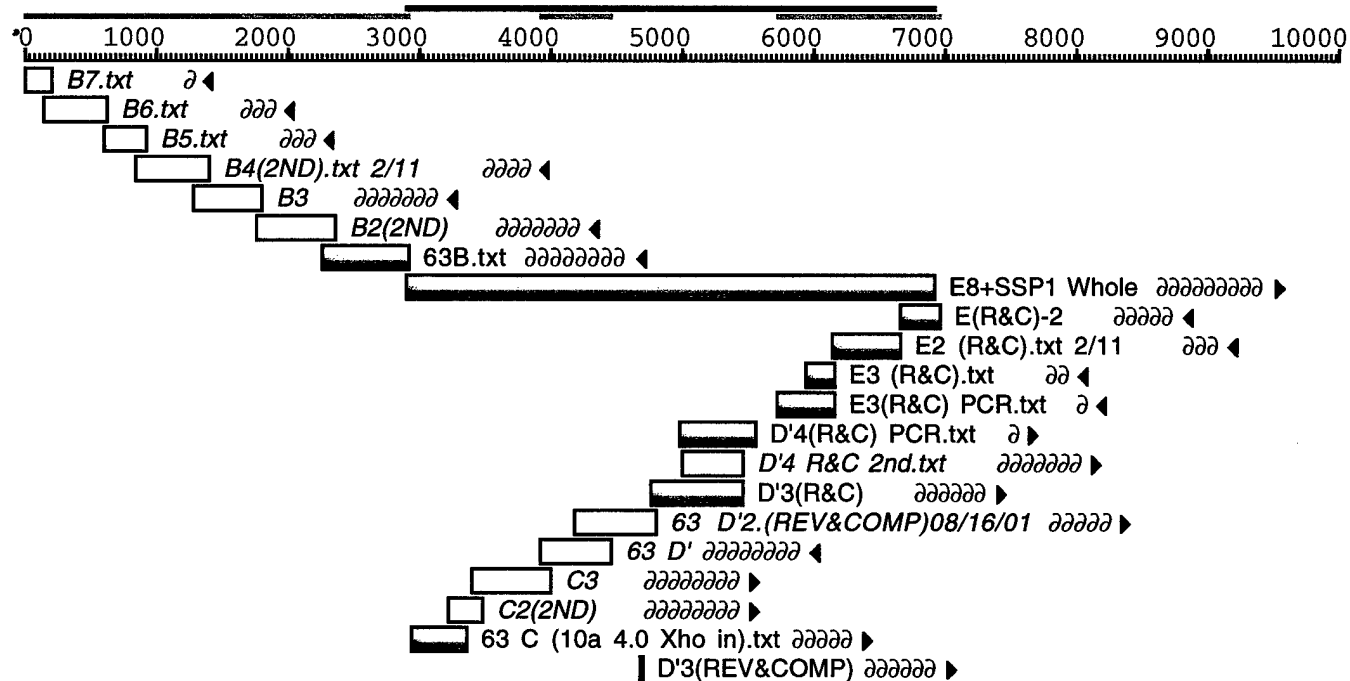
References

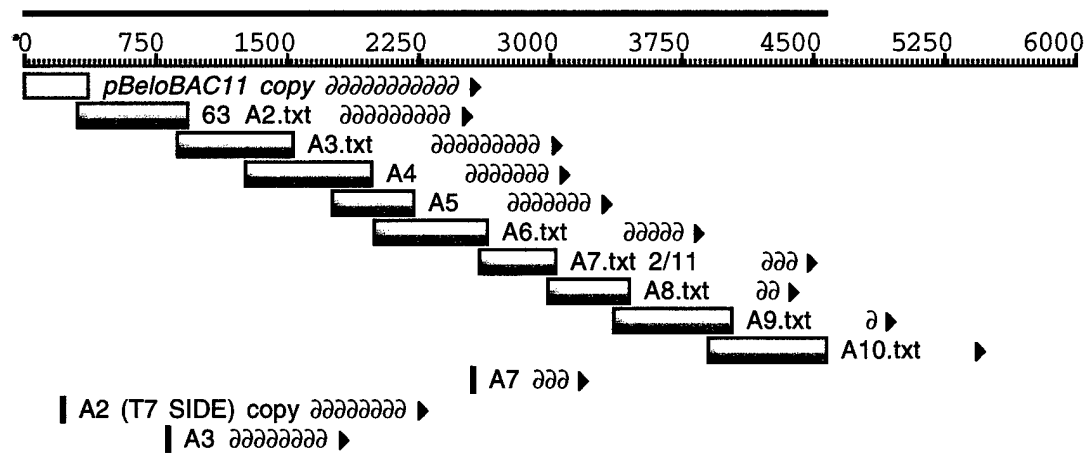
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- Yang, X.W., Model, P. and Heintz. Homologous recombination based modifications in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nature Biotechnology* **15**:859-865, 1997.

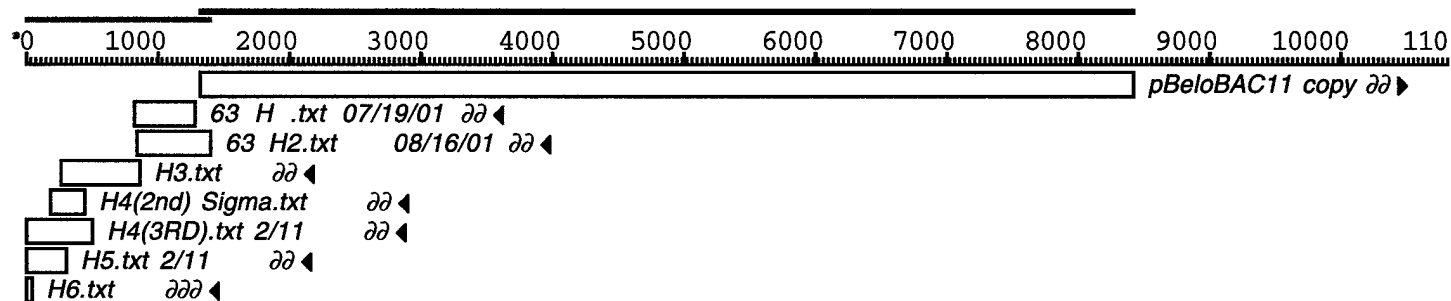
Appendix A: Sequencing analysis of Scn10a BAC clone.

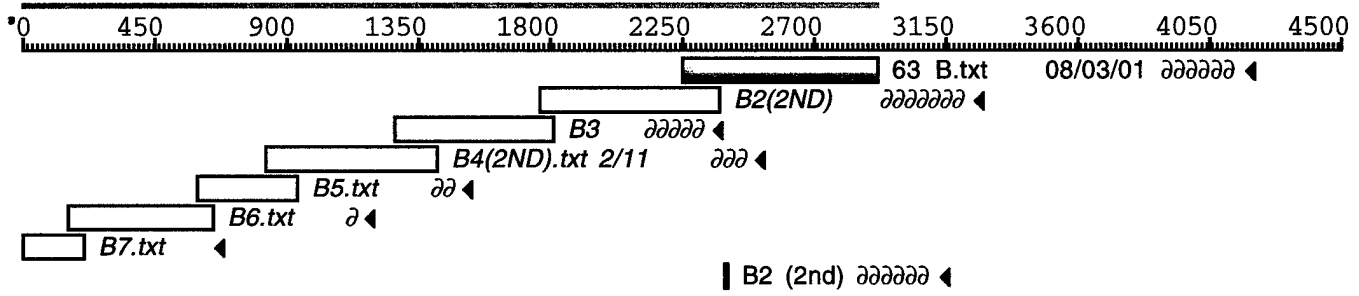


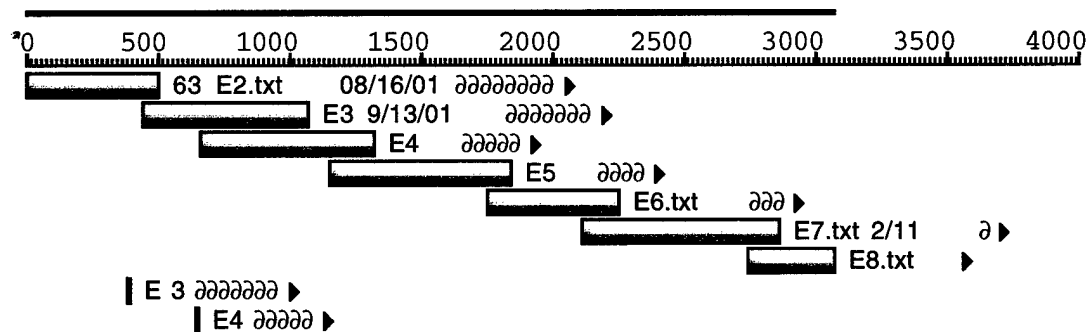
The alignment contigs are in relation to their point of origin as indicated by the horizontal arrows in the above schematic. The alignment data was generated by successive rounds of DNA sequencing.

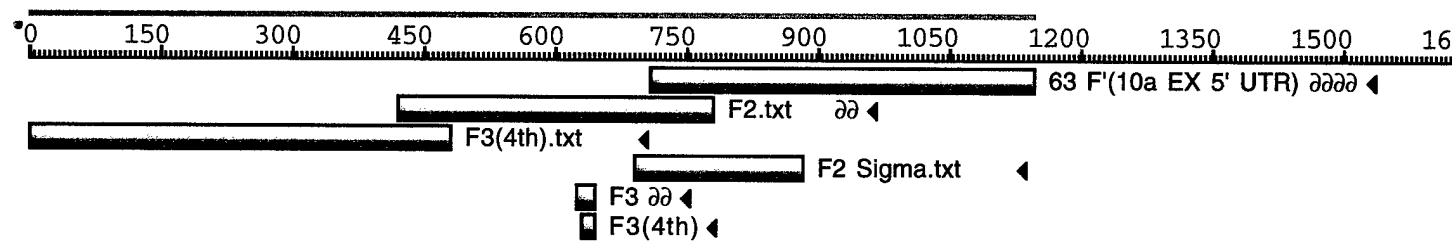




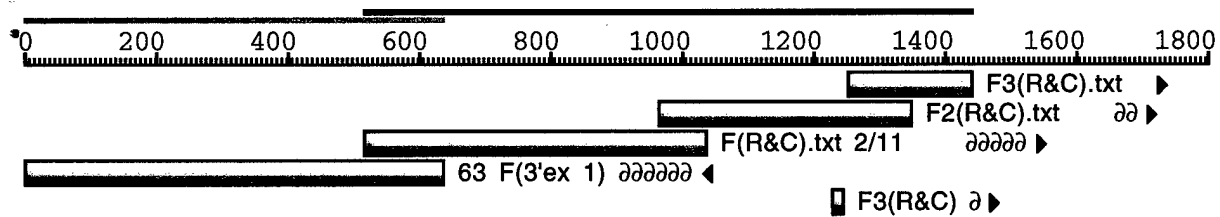


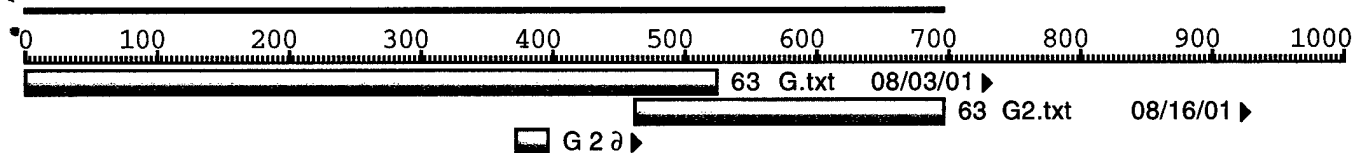






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E8-E8 del primers Map

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